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Reproductive modes, ploidy distribution, and supernumerary chromosome frequencies of the flatworm *Polycelis nigra* (Platyhelminthes: Tricladida)

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Key words: flatworm, *Polycelis*, polyploidy, pseudogamous parthenogenesis, supernumerary chromosome, Tricladida

Abstract

The hermaphroditic flatworm, *Polycelis nigra*, is characterized by two reproductive biotypes which differ with respect to ploidy; sexual individuals are diploid (n = 8, 2× = 16) and pseudogamous parthenogenetic individuals are polyploid (typically 3×). We have collected and karyotyped individuals from 15 sampling sites (13 in mid to northern Italy, one in Great Britain and one in The Netherlands). We found that biotypes can exist alone or in sympatry, and identified purely diploid, mixed diploid-polyploid, and purely polyploid populations. Karyotype data show that in addition to the normal autosome complement, B chromosomes of differing morphology as well as stable aneuploid chromosomes (extra-A) were found almost exclusively in polyploids (11 of 12 sites). We extensively sampled Lago di Toblino (northern Italy), a pure polyploid population characterized by a submetacentric to metacentric, mitotically stable B chromosome, as well as a stable extra-A chromosome. Here, individuals having 1–3 B chromosomes were more abundant (61%) than those having no B's, implying that B chromosome infection has little detrimental effect when occurring in low numbers. Furthermore, 66% of individuals from this population possessed extra-A chromosomes, although it is unclear whether these elements are aneuploid autosomes or B chromosomes of different morphology. The ubiquity of these chromosomes, within asexuals in particular, is suggestive of a correlation between the origination of the elements and the evolution of polyploidy, or may reflect increased tolerance of parthenogenetic genomes to aneuploidy.

Introduction

Parthenogenetic taxa are typically characterized by elevated ploidy relative to their sexual relatives (Dawley and Bogart, 1989; Menken et al., 1995; Stenseth et al., 1985; Turgeon & Hebert, 1994). While the genetics regarding the relation between polyploidy and parthenogenesis are unclear, the stable coexistence of the two phenomena in taxa separated by deep phylogenetic gaps implies interdependence, and more specifically, that polyploidy is required for stable parthenogenesis (Suomalainen et al., 1987). Asexual polyploid organisms have greater tolerance to mutation load (dilution of harmful mutations) or increased genetic plasticity compared to clonal diploids. As such, diploid parthenogens which hypothetically span the evolutionary transition from diploid sexual to stable polyploid parthenogen are characteristically ephemeral, and this is supported by their relative absence from studies of parthenogenetic taxa (Suomalainen et al., 1987). In contrast, polyploid parthenogens are relatively common and successful (Menken et al., 1995; Suomalainen et al., 1987; Turgeon & Hebert, 1994), and can be extremely old (Hedges et al., 1992; Spolsky et al., 1992).

In animals, parthenogenesis and polyploidy represent derived conditions relative to diploid sexuality (Suomalainen et al., 1987). Multiple origins of parthenogenetic lineages can lead to complex and overlapping distributions of asexual lineages relative to those of their diploid sexual ancestors (Ó Foighil & Smith, 1995; Richards, 1973). Determining the number of clonal lineages at intra- and interpopulational levels has relied on detection of allelic phenotypes using isozyme (Turgeon & Hebert, 1994), protein (Turner et al., 1983), and mtDNA markers (Densmore et al., 1989), as well as histocompatibility complex analysis (Angus & Schultz, 1979; Moore & Eisenbrey, 1979). Chromosomes have also been employed (Turner et al., 1980), although to a much lesser degree as they do not exhibit sufficient variability to differentiate between clonal lineages within the same taxon. Alternatively, chromosome morphology has been informative for identifying the parental taxa of parthenogenetic lineages which have arisen through hybridization between sibling species (Turner et al., 1980).

Polyploidy, aneuploidy, and supernumerary chromosomes are characteristic of the freshwater flatworm group Tricladida (Benazzi & Benazzi Lentati, 1976; Canovai, 1989; Canovai & Galleni, 1988; Lepori, 1950; Melander, 1950). Taxa within this group are hermaphroditic, and commonly demonstrate multiple reproductive biotypes which differ with respect to ploidy (Benazzi & Benazzi Lentati, 1976; Beukeboom et al., 1996b). We are currently studying one member of this group, *Polycelis nigra*, which has diploid sexual and polyploid (typically triploid and sometimes tetraploid) pseudogamous parthenogenetic (= gynogenetic) biotypes (Benazzi & Benazzi Lentati, 1976; Lepori, 1950). By definition, fertilization by sperm is necessary in order to trigger zygote development in pseudogamous parthenogens, but the paternal set of chromosomes is subsequently eliminated. This species inhabits ponds, lakes and quiet reaches of lowland streams (Den Hartog, 1962; Reynoldson, 1978), where different biotypes can be found alone or in syntopy. Outcrossing appears to be the rule, although occasional self-fertilization has been observed after prolonged isolation in the laboratory (Benazzi, 1952; Beukeboom, unpublished.). Limited hybridization between biotypes may be possible since parthenogens produce fertile polyploid sperm which could fertilize the haploid eggs of sexual individuals to generate polyploid offspring. In this way it is hypothesized that new parthenogenetic lineages can be continually produced (Benazzi & Benazzi Lentati, 1976).

The haploid chromosome complement of *P. nigra* is $n = 8$ (Benazzi, 1963; Benazzi & Benazzi Lentati, 1976; Le Moigne, 1962), and this basic chromosome number has been documented in individuals from several European localities (Lepori, 1949, 1950, 1954a,b, 1955), including Italy (Canovai, 1989; Canovai & Galleni, 1988; Canovai et al., 1985), southern Sweden (Melander, 1963), the Netherlands (De Vries, 1985 and Van der Velde), and France (Dutrilleux & Lenicque, 1971; Le Moigne, 1962; Lepori, 1954a). In addition to the basic karyotype, supernumerary (B) chromosomes (Jones & Rees, 1982) of variable morphology (Canovai, 1989; Canovai et al., 1985) have been described in diploids and polyploids from different populations. Aneuploidy has been reported only from polyploids (Lepori, 1950).

Previous karyological studies did not report sample sizes, and as such no estimation can be made regarding inter- and intrapopulational proportions of different biotypes. Here, we present data on the distribution and frequencies of biotypes in *P. nigra*. They have been collected as part of our work on the reproductive biology of hermaphroditic fresh water flatworms, and assist our understanding of the evolution of sexual and parthenogenetic forms in these organisms. In addition, we document geographic distribution of different supernumerary chromosome forms and investigate their association with reproductive modes characteristic of this species.

Material and methods

All material was collected from 1993 to 1996. Stones were picked from the water's edge of lakes or streams, and worms were rinsed from under-surfaces using a squirt bottle. Worms were transported in brown plastic containers to the laboratory. We collected mainly in northern Italy, but some other European localities were also sampled. Our sample sites included ones reported in the literature as well as new localities not previously investigated. In some cases, different sites within the same lake or stream were sampled. Data from sites visited more than once were pooled.

Karyotypes were derived from regenerated blastemas or whole young animals according to a modified protocol from Redi et al. (1982). After colchicine treatment (0.15% for 2–4 hours at room temperature), tissue was hypotonically shocked in distilled water

for 20 minutes and fixed in ice-cold Carnoy's solution (3 parts methanol to 1 part glacial acetic acid). Squash preparations of preserved tissue were made by dispersing nuclei in 45% acetic acid under coverslips. Slides were examined and photographed under phase-contrast on an Olympus BHS or BH2-RFCA microscope.

Results

We collected and karyotyped a total of 786 *P. nigra* from 13 localities in Italy, one locality in Great Britain and one in The Netherlands (Figure 1, Table 1). All specimens had a basic (haploid) chromosome complement of $n = 8$ (Figure 2), and no distinct differences in chromosome arm lengths could be detected among the populations. Italian populations ranged from purely diploid (Lago di Garda, La Fattoria 1) to mixed diploid-triploid (Lago di Garlate, Lago di Lamar, Lago di Caldonazzo, Lago di Levico and La Fattoria 2) to purely polyploid (Lago di Como, Arco, Lago d'Iseo, Lago di Toblino, Pontatetto and Massa Pisana; Table 1). Tetraploid individuals were relatively uncommon and were found only in three purely polyploid populations. All individuals from Sheffield (Great Britain) were triploid, and all from Nederhorst den Berg (The Netherlands) were diploid (Table 1).

Two types of supernumerary chromosome were observed, typical B chromosomes and extra elements resembling autosomes ('extra-A' from here on; Figure 2). B chromosomes were readily identifiable as they were significantly smaller than any of the autosomes. Extra-A chromosomes, which were inferred through chromosome counts, were never observed among diploids, but frequently among triploids in virtually every sampled population (Table 1). These chromosomes could not be morphologically distinguished from the standard chromosomes (Figure 2B, C), and thus we consider this a form of aneuploidy. In addition to the normal triploid karyotype ($3\times = 24$), we found a common aneuploid karyotype of 25 ($3\times+1$) chromosomes and a less frequent 26 ($3\times+2$) or 33 ($4\times+1$) chromosome karyotype (Table 1). Aneuploidy was never manifested in a karyotype containing less than the triploid complement of 24 and their numbers usually did not vary between cells within an individual. This suggests that they represent a second class of mitotically stable supernumerary element. Extra-A chromosome frequencies ranged from 0% to 66% in the north (Lago di Garda and Lago di Toblino) to 72% in the Pisa region (La Fattoria 2).

Of the B chromosomes found in our sampling, two forms could be identified with respect to their morphology and association with specific genotypes (Table 1). These could further be differentiated into a tentative disjunct north/south distribution separated by the Po valley (Italy). North of the Po valley, B's were metacentric (Figure 2C), associated only with polyploid genotypes, mitotically stable in Lago di Como, Lago d'Iseo, Arco, Lago di Toblino and Lago di Caldonazzo, and unstable in Lago di Garlate. South of the Po valley, telocentric (Figure 2D) and metacentric forms were identified in both diploid and polyploid individuals, and were typically unstable mitotically (Table 1). In one case, two different forms of southern B's were found within single nuclei.

On the population level, frequency of B-carrying individuals showed great variability. North of the Po Valley, population frequencies of B-carrying individuals ranged from 0% (Lago di Lamar, Lago di Garda, Lago di Levico) to as high as 90% (Arco; Table 1). South of the Po Valley, B frequencies ranged from 28% (La Fattoria 2) to 100% (Massa Pisana; Table 1). In populations exhibiting mitotically stable B and extra-A chromosomes, B chromosome numbers varied from 1 to 3 and extra-A numbers from 1 to 3 per individual (one exceptional individual from Lago di Toblino had 5 extra A-like chromosomes), although individuals with one or two elements were most common. Figure 3 shows frequencies of supernumerary chromosomes in Lago di Toblino, which was studied in detail (494 individuals karyotyped). In this lake, individuals having both extra-A and B chromosomes or lacking both were more frequent than those having only one of the supernumerary elements (Figure 3).

Discussion

Ploidy forms and geographical distribution

After collectingworms from 50 localities, Lepori (1955) reported *P. nigra* from northern Italy, southeastern France and Wales. Based on these data, he concluded that *P. nigra* occurs above the 4° C January isotherm, which extends from north to south over central Great Britain and central France, then west to east across the Alps and further south over former Yugoslavia. However, this 4° C isotherm hypothesis (Lepori, 1955) has to be negated, as *P. nigra*

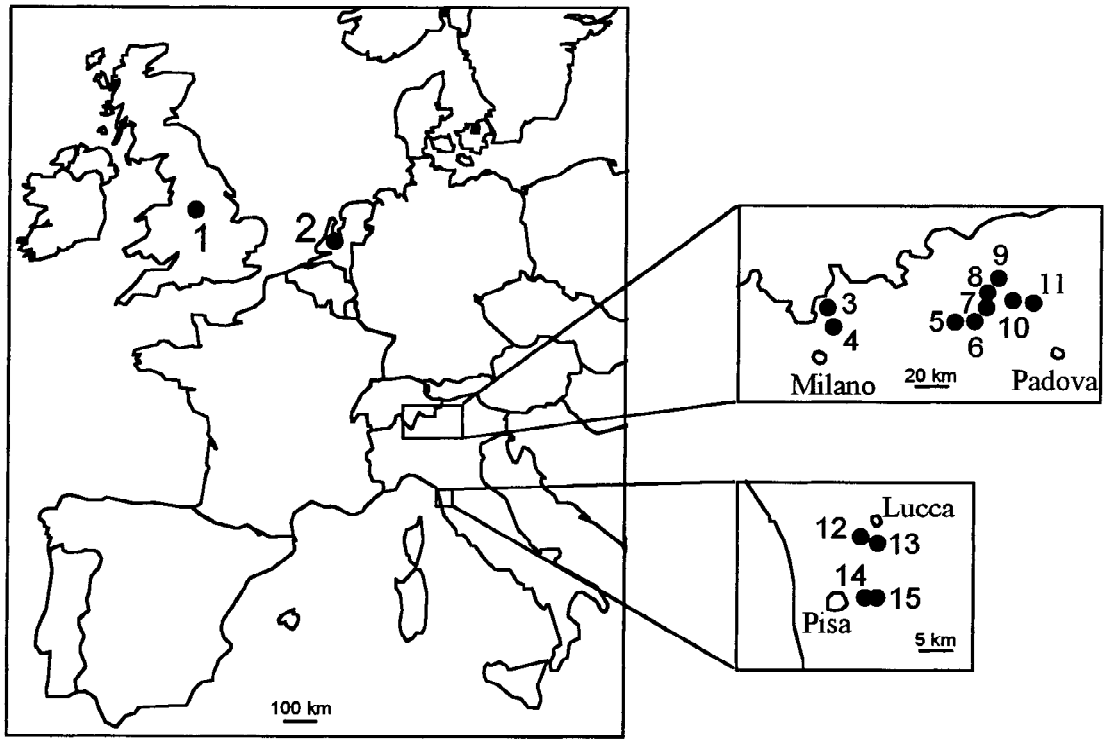

Figure 1. Sample localities of *Polycelis nigra* in western Europe. Numbers represent the following localities: (1) Sheffield (Great Britain), (2) Horstermeer, near Nederhorst den Berg (The Netherlands), (3) Lago di Como, near Santa Maria, (4) Lago di Garlate, near Olginate, (5) Lago d'Iseo, (6) Lago di Garda, near Peschiera, (7) Arco, (8) Lago di Toblino, (9) Lago di Lamar, (10) Lago di Caldonazzo, (11) Lago di Levico, (12) Pontetetto, near Lucca, (13) Massa Pisana, near Lucca, (14) La Fattoria 1, near Pisa, (15) La Fattoria 2, near Pisa (all Italy).

has subsequently been reported from the Netherlands (Biersma & Wijsman, 1981; Den Hartog, 1962; De Vries 1985; Steenbergen, 1993; Van der Velde; this study), Belgium (Van Oye, 1941), Luxemburg (Hoffmann, 1964), France (Biersma & Wijsman, 1981; Pattee & Gourbault, 1981) and Germany (Heitkamp, 1979), while their absence from Scandinavia appears to be real (Den Hartog, 1962; Hansen-Melander et al., 1954).

Relatively little is known about the distribution of ploidy forms and associated reproductive modes in *P. nigra*. Schleip (1907) first reported 8 bivalent chromosomes in spermatocytes from *P. nigra* collected in Germany. Together with a population from Luxemburg (Hoffmann, 1964) and Switzerland (Melander, 1963), as well as our sample from the Netherlands, these are the only reports of sexual diploids ($2\times$ = 16) outside of Italy. Our data confirm the abundance and ubiquity of *P. nigra* in Italy, where pure diploid, pure polyploid ($3\times$ and $4\times$) and mixed populations occur, and corroborate the data of Canovai (1989), Canovai & Galleni (1988), Canovai et al. (1985), Lepori (1949, 1950), and Melander (1963). We report for the first time polyploids from Great Britain. The only previously reported polyploid population outside of Italy is from the river Var near Nice, France, which is characterized by 36 chromosomes. This led Lepori (1954a) to conclude that *P. nigra* had a haploid complement of 6 ($2\times$ = 12), as the river Var population was interpreted as hexaploid ($6\times$ = 36) and Italian populations with 24 chromosomes as tetraploid. However, the most consistent haploid chromosome number in *P. nigra* is clearly 8 (Figure 2), and individuals with 24 chromosomes are thus triploid forms. The river Var population likely represents a hyperpolyploid form (Benazzi, 1963; Benazzi & Benazzi Lentati, 1976), or perhaps an undescribed species with similar external morphology to *P. nigra*.

Distribution and frequencies of supernumerary chromosomes

Supernumerary chromosomes were abundant in most *P. nigra* populations, and our data indicate that they

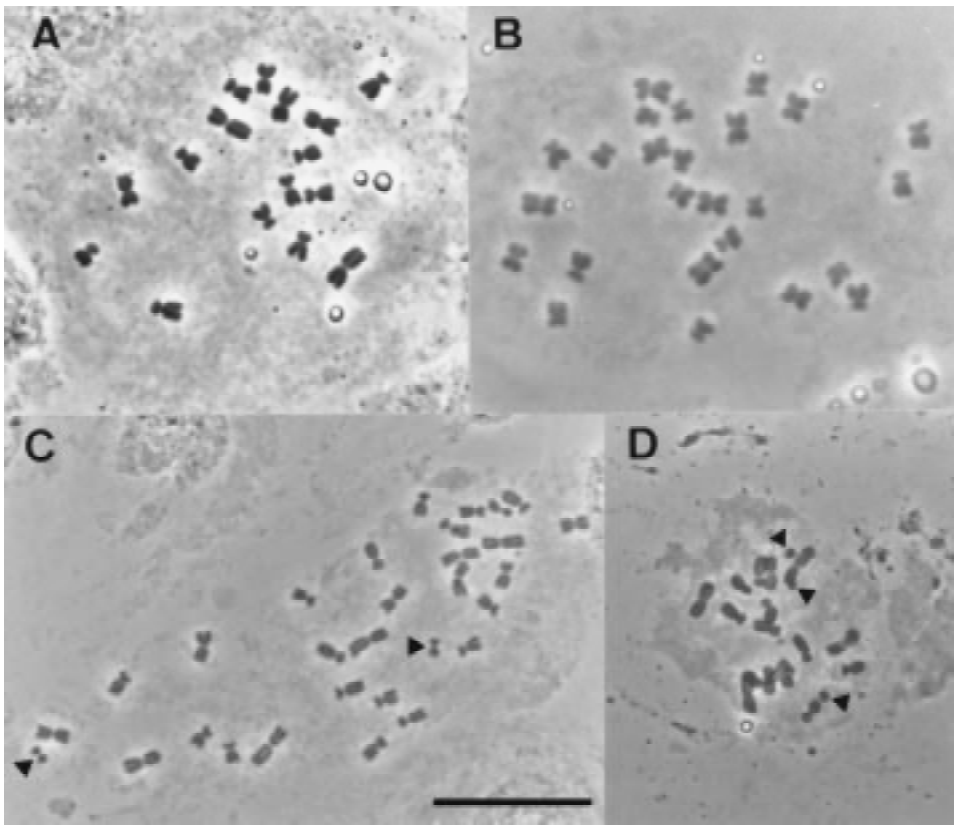

Figure 2. Metaphase chromosome spreads of *Polycelis nigra (a)* diploid (n = 8, 2× = 16), *(b)* triploid plus one extra-A chromosome (3× = 24 + 1A), *(c)* triploid plus three extra-A and two submetacentric B chromosomes (3× = 24 + 3A + 2B), and *(d)* diploid plus three telocentric B chromosomes (2× = 16 + 3B). Arrows indicate the B chromosomes. The extra-A chromosomes are indistinguishable from the autosomes.

occur almost exclusively in pseudogamous parthenogenetic polyploids (Table 1). The smaller of the two supernumerary elements is by definition a B chromosome, although this nebulous term implies no specific set of criteria other than its appearance, degeneracy, and independence relative to the autosome set (Jones & Rees, 1982; Beukeboom, 1994). Based upon morphology and mitotic stability (or lack of), we consider there to be at least two distinct B chromosomes within our sampled populations. The first is the small, mitotically unstable telocentric form characteristic of three of our sampled populations near Pisa, Italy (Table 1), and has been previously described from other populations within the same region (Canovai, 1989). This particular element was the most distinct of the B chromosomes we have examined. It was frequently mitotically unstable, accumulated to relatively high (up to 5) intranuclear frequencies, was found within diploid and polyploid genomes, and was occasionally present with a metacentric B within the same nucleus. The metacentric B was infrequent within southern populations, and considering its sporadic intranuclear accompaniment with the telocentric B, we hypothesize that it may be an isochromosome form of the latter. We further identified a second B chromosome type, a mitotically stable submetacentric to metacentric element which occurred only in polyploids. This B was considerably more common than the telocentric (Pisa) form and was found in most populations north of the Po valley, and resembled the B identified in Sheffield (Great Britain) individuals.

Metaphase chromosome counts under phase contrast enabled us to establish the presence of extra A chromosomes, although this method had insufficient resolution to pinpoint any specific chromosome as the supernumerary element. Mitotic stability and relative ubiquity of extra-A chromosomes in polyploids suggests that it is neither a simple aneuploid phenomenon, nor an artefact induced by colchicine treatment (Ribas et al., 1988). Such aneuploidy has been observed in many planarian species (Benazzi & Benazzi Lentati, 1976; Benazzi Lentati, 1970;

Table 1. Karyotypes, ploidy levels and chromosome frequencies of *Polycelis nigra* populations. Refer to Figure 1 for location of collection sites

Locality	No. individuals	Ploidy	Frequency	Extra-A	Meta-centric B	Telo-centric B
Sheffield (UK)	4	**3**×	1.00	0.50	0.50	–
Nederhorst den Berg (NL)	10	**2**×	1.00	–	–	–
Lago di Como(I)	33	**3**×	0.97	0.18	0.54	–
		4×	0.03	–	0.03	–
Lago di Garlate (I)	29	**2**×	0.24	–	–	–
		3×	0.76	0.13	0.23[1]	–
Lago d'Iseo (I)	13	**3**×	1.00	0.54	0.63	–
Lago di Garda (I)	10	**2**×	1.00	–	–	–
Arco (I)	10	**3**×	0.80	0.50	0.80	–
		4×	0.20	–	0.10	–
Lago di Toblino (I)	494	**3**×	0.99	0.65	0.60	–
		4×	0.01	0.01	0.01	–
Lago di Lamar (I)	10	**2**×	0.80	–	–	–
		3×	0.20	–	–	–
Lago di Caldonazzo(I)	93	**2**×	0.92	–	–	–
		3×	0.08	–	0.02	–
Lago di Levico (I)	38	**3**×	0.32	–	–	–
		3×	0.68	0.13	–	–
Pontetetto (I)	10	**3**×	1.00	–	0.80	–
Massa Pisana (I)	7	**3**×	1.00	0.28	–	1.00
La Fattoria 1 (I)	10	**2**×	1.00	–	0.10	0.90[1]
La Fattoria 2 (I)	14	**2**×	0.14	–	–	0.07[1]
		3×	0.86	0.72	–	0.21

[1] Mitotically unstable

Canovai, 1989; Dahm, 1958; De Vries et al., 1984; Le Moigne, 1962; Lepori, 1950; Lepori 1954b; Lepori and Pala, 1982; Melander, 1963; Oki et al., 1981) including *P. nigra* (Lepori, 1950). Extra-A elements could either be polysomics, or another type of B chromosome that morphologically resembles the standard (A) chromosomes, and we are presently investigating these alternative hypotheses using chromosome banding techniques (Sharbel et al., 1998).

In Lago di Toblino, frequency of individuals with 1 or 2 supernumerary elements is greater than that of individuals with none. Such a distribution implies that low-level supernumerary chromosome 'infection' is tolerated by individuals. Indeed, animals with 1 or 2 B chromosomes were found to have almost similar fitness to individuals lacking Bs (Beukeboom et al., 1998). Conversely, frequency of individuals having 3 or more supernumerary chromosomes drops off rapidly (Figure 3), implying a disadvantage associated with having greater numbers of Bs. In addition, animals with either none or both supernumerary types (B and extra-A) occurred at significantly higher frequencies than individuals with only one of the supernumeraries. This could mean that their origin is somehow correlated or that there is some advantageous synergism between the B and extra-A chromosomes. Alternatively, lineages may exist within Lago di Toblino that differ in resistance to supernumerary chromosome infection.

Origin and transmission dynamics of supernumeraries

Differences in mitotic stability and morphology between the B chromosomes found north and south of the Po valley are suggestive of independent interpopulational origins. As univalent elements which do not synapse with the autosomes, B's do not undergo recombination and as such can degenerate rapidly in comparison to autosomes (Green, 1990). B chromo-

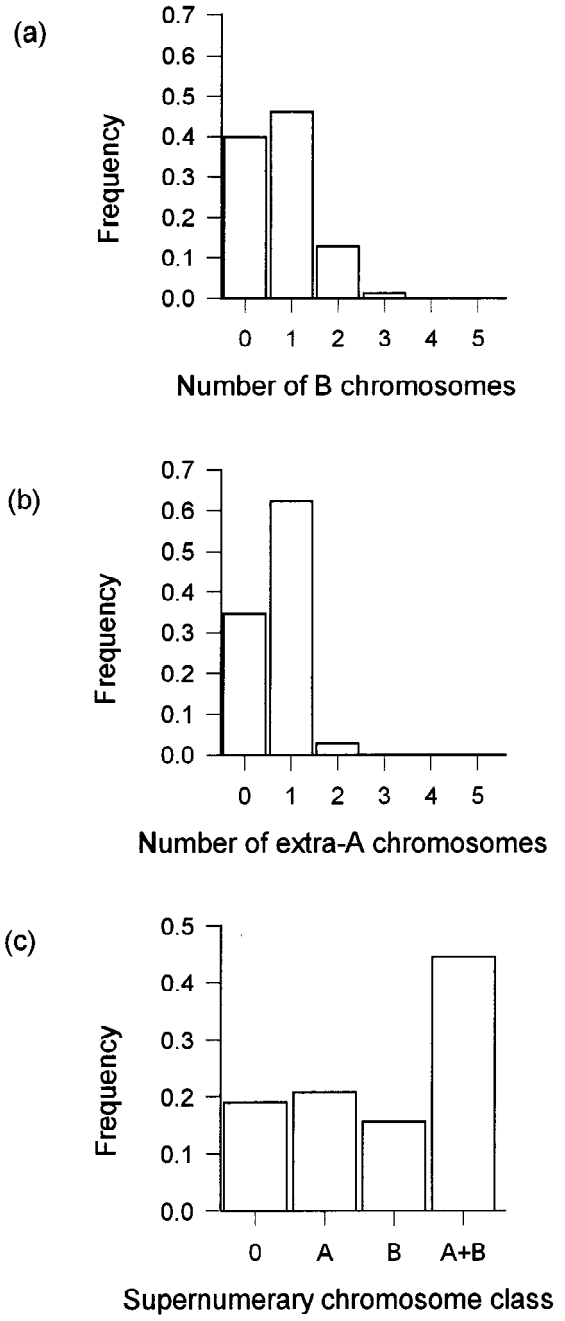

Figure 3. Frequencies of mitotically stable supernumerary chromosomes per individual in Lago di Toblino; (a) B chromosomes, (b) extra-A chromosomes and (c) both supernumeraries. The frequency of individuals having none or both of the supernumeraries is significantly higher than expected under random association ($\chi^2 = 24.85$, $p < 0.0001$).

somes in different parthenogenetic lineages therefore represent independently evolving entities, and it is unlikely that they have converged upon similar intrapopulational morphologies due to the stochastic nature of their degeneration. Alternatively, it is possible that B chromosome derivation occurred only once followed by its spread through *P. nigra* populations. In this case, variability in mitotic stability of the B's may stem from interpopulational differences in genetic tolerance to B chromosome infection, as has been shown for the grasshopper *Eyprepocnemis plorans* (Camacho et al., 1996). In addition, B chromosome proliferation could have been facilitated by occasional paternal inheritance. Correlative evidence from Lago di Toblino (Italy) indicates that B's can sometimes escape paternal chromosome expulsion in crosses between pseudogamous parthenogens (Beukeboom et al., 1996a).

A similar collection of reproductive modes and associated karyotypes is known from the dandelion *Taraxacum* (Menken et al., 1995; Richards, 1973). In this complex, the primitive karyotype is a diploid condition lacking accessory chromosomes, and thus polyploidy, parthenogenesis, and the evolution of supernumerary elements represent derived conditions. The similar association between ploidy, supernumerary chromosomes and reproductive mode in *P. nigra* lead us to believe that B chromosome origination may be associated with polyploidy and possibly parthenogenesis. It is known that the process of pseudogamous parthenogenesis is not always perfect, and traces of paternal chromatin have been observed during early embryogenesis (Benazzi Lentati, 1970). B chromosomes may actually have originated as incompletely eliminated autosomes. A cytologically similar phenomenon where (parts) of autosomes appear to survive after elimination of the paternal genome is known from cytoplasmic incompatible crosses (Reed & Werren, 1995; Ryan et al., 1985, 1987). In addition, their persistance in polyploid lineages may be more likely because gametogenesis consists of an endoduplication of all chromosomes followed by a normal reduction division (Benazzi & Benazzi Lentati, 1976). This appears to lead to stable vertical transmission, whereas an accumulation mechanism would be required for maintenance in sexual lineages.

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